MULTI-VARIANT DIFFERENTIATION OF HEALTHY AND CANCEROUS WHITE BLOOD CELLS TO PROGESS THE DIAGNOSIS OF LEUKEMIA

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MULTI-VARIANT DIFFERENTIATION OF HEALTHY AND CANCEROUS WHITE BLOOD CELLS TO PROGESS THE DIAGNOSIS OF LEUKEMIA

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To Mike and Lisa Crawford, who certainly gave me big shoes to fill.

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LIST OF SYMBOLS AND ABBREVIATIONS

WBC	White Blood Cell
RBC	Red Blood Cell
AFM	Atomic Force Microscopy
CBC	Complete Blood Count
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
CLL	Chronic Lymphoblastic Leukemia
CML	Chronic Myeloid Leukemia
LSC	Lymphoid Stem Cell
MSC	Myeloid Stem Cell

SUMMARY

Every three minutes, someone in the United States is diagnosed with blood cancer, the most common of which being Leukemia, or the cancer of white blood cells [1]. Distinguishing between healthy white blood cells and leukemia cells has proven to be difficult because the physical and visual similarities between the two. Unlike tumorous cancers, leukemia cells are much more difficult to distinguish and characterize in a clinical setting because of the physiologic nature of the disease. Current diagnostic methods like flow cytometry are known to be time-consuming and costly, creating a crucial need to more effectively identify white blood cells (WBCs) from leukemic cells.

The purpose of this study is to use biomechanical markers to characterize the distinct properties of healthy WBC's and the different types of leukemia. By measuring multiple biomechanical characteristics of each type of cell, each cell type will be able to be narrowed down into a cluster that is representative of the biomechanical characteristics distinctive to that cell type. As we know, it is difficult to distinguish differences in leukemic and healthy WBC's by a factor of size alone, but adding additional biophysical parameters may lead to improved identification of the pathological condition. Our proposal intends on using four distinct biomechanical parameters to complete our analysis: size, elastic modulus, and both slow and quick viscoelastic response time constants, tau1 and tau2. It has been shown that distinct differences in these specific characteristics do exist between healthy and cancerous WBC's [2,3], so by analyzing these parameters against each other, we hope to gain a more complete understanding of each of these cell types' specific biomechanical blueprint for applications in clinical diagnostics.

CHAPTER 1

INTRODUCTION

Leukemia

Leukemia is a blood disorder that affects over one million people in the United States alone. Leukemia is a cancer of white blood cells, where genetic mutations cause unregulated cell cycle control and affect the normal division and proliferation of affected cells. The leukemic cells do not undergo normal cell apoptosis when damaged, thus accumulating and overcrowding the normal blood cells [1]. Because of the wide variety of white blood cells (WBC's) in the human body, leukemia is very different from other cancers in the breadth of cases, affecting patients anywhere from young children to the elderly. Also unlike most cancers, age, race, and lifestyle habits, and background do not exempt anyone from being at risk for the disease, and accounts for over 50,000 new cancer cases annually [1]. Because it does not form tumors, leukemia is not considered "metastatic", but it does form dangerous accumulations in the lymph nodes, spleen, and brain [1].

White Blood Cell Physiology and Leukemia Typing

When considering typing leukemia, the physiologic origin of the affected cells is the distinguishing characteristic between the types. Depending on the origin, one of four main types may arise, and each types carries similar characteristics and symptoms. In the pluripotent form, a blood stem cell may give rise to all components of blood cells, including all white and red cells, and platelets [1]. A blood stem cell further differentiates into a myeloid stem cell (MSC), or a lymphoid stem cell (LSC). As the same suggests, an MSC originates in the bone marrow, while an LSC will originate in the lymph nodes. The

MSCs and LSCs further differentiate into myeloblasts and lymphoblasts, respectively (Figure 1). Each of these two cells can become cancerous, forming either myelogenous or lymphoblastic leukemia. Each of these morphologies give rise to either a chronic or acute cancer. Acute leukemias have a rapid onset, characterized by abnormally high accumulations of mutated cells and a quick deficiency in normal lymphoblast functioning. Symptoms may include fatigue, bruising, and extreme immunosuppression and susceptibility to disease [1]. Chronic leukemias, however, progress slowly, and the cancerous cells function almost as well as their healthy WBC counterparts. Abnormal blood tests may be preliminary indicators of chronic leukemia, and patients may not know they are infected due to lack of noticeable symptoms [1]. The combination of the cellular origin and the onset of the disease gives rise to the four main leukemia types: Acute Myeloid Leukemia (AML), Acute Lymphoblastic Leukemia (ALL), Chronic Myeloid Leukemia (CML), and Chronic Lymphoblastic Leukemia (CLL). AML accounts for 36% of all new cases of leukemia, but is by far the most lethal with a bleak 25.4% 5-year survival rate [1,4]. ALL accounts for over 75% of all new cases in children, and the risk of contraction is highest amongst children under the age of 5 [1,4]. CML has a relatively low 5-year survival rate of 59.9%, and accounts for 11% of all new cases [4]. CLL is relatively common, accounting for 30% of new cases, but carries a relatively high survival rate of 83.5% [4]. This study chooses to investigate three of the four main cancer types: ALL (childhood leukemia), AML (the most lethal), and CML (the most common in elderly patients) [1,4]. Jurkat was the immortalized model cell line for ALL, HL60 was the model for AML, and K562 was the model for CML. These cell types are commonly seen throughout the literature. Neutrophils were chosen for the healthy myeloid cell

model, which make up 80-90% [5] of myeloid cells. Lymphocytes were chosen as the lymphoblastic cell model.

Diagnostics and Treatment

When someone is thought to have leukemia, a series of tests are performed to confirm the diagnosis, determine the type of leukemia present, and develop a prognosis and treatment plan. Blood tests are used in conjunction with bone marrow tests to develop a complete diagnosis, starting with a complete blood count (CBC) [5]. Blood is taken form a peripheral vein and the amount of each type of WBC, along with RBCs and platelets, are determined. Depending on the type of leukemia present, certain white blood cells will be uncharacteristically high or low. A peripheral blood smear may be completed, which involves placing a drop of blood of a microscope slide for viewing [5]. Morphological changes in WBCs are noted. If blood tests cannot rule out leukemia, a bone marrow biopsy is performed. In a bone marrow collection procedure, a local anesthetic is given, and a small needle is inserted into the back of the hip. The first sample collected is usually done through a bone marrow aspiration, where a small amount of liquid bone marrow is expunged though a syringe. If needed, a bone marrow biopsy is performed, in which a larger needle is used to extract larger, solid amounts of marrow. Even with properly applied local anesthetic, most patients report feeling pain upon the biopsy needle entering the hip bone.

Indications of diseased-state cells

Historically, biochemical markers have been utilized to determine cancerous potential of cells. Gene expression or suppression have been widely studied as indicators of cancer, as well as different chemical markers present on the surface of cells. More recently, however, the mechanical properties of cells have given researchers more of an insight into how disease affects cellular structure and function, and perhaps even using it

as a marker to predict diseased versus healthy state cells. White blood cells and leukemia cells have been of particular interest in the field of cellular biomechanics, as it is presumed that the mechanics of the two morphologies differ [2]. Researchers have used optical trapping techniques to measure the mechanical properties of neutrophils [6]. More specifically, studies were conducted to measure neutrophil protrusional stiffness, defined by the loading rate in relation to stiffness. The dependence of these two factors indicated a viscoelastic character of WBCs [6], and assumptively their cancerous counterparts. Studies have shown that the relative stiffnesses of WBC morphologies and cancer lines are significantly different from each other, and even different from their own cell type at different phases of differentiation [7]. In other studies, the Atomic Force Microscope (AFM) proved to be a useful tool for measuring components of blood, including both neutrophils and platelets [8].

CHAPTER 2

MATERIALS AND METHODS

Cancer Cell Culture

Jurkat cells were cultured in 20% Fetal Bovine Serum (FBS), 1% Pennicilin-Streptomycin (Pen-Strep), and 79% Roswell Park Memorial Institute (RPMI) media base. K562 cells were cultured in 10% FBS, 1% Pen-Strep, and 89% Dulbecco's Modified Eagle's Medium. HL60 cells were cultured in 20% FBS, 1% Pen-Strep, and 79% DMEM media base. Media materials were obtained from Sigma Aldrich, and were stored in a culture refrigerator. Immortalized cell lines were obtained from American Tissue and Cell Culture. All cell lines were grown in suspension. Cells were passed every 2-3 days, and stored in an incubator at 37 degrees Celsius.

Healthy Cell Isolation

Healthy white blood cells (WBCs) were harvested from samples of healthy donor blood. Donor blood was taken in 3 ml samples via venipuncture of a peripheral vein. Blood was stored in a blood collection tube coated with EDTA/anticoagulant. Whole blood was immediately centrifuged with a red blood cell lysis buffer in a concentration of 25%. After 10 minutes of centrifugation, DPBS was added to stop the RBC lysis reaction, aspirate was collected, and remaining WBC pellet was again centrifuged with red blood cell lysis buffer. The samples were then placed in a centrifuge tube with 3 ml of DPBS, and stored in an incubator at 37 degrees Celsius until plating.

Cell Plating and Preparation

Both healthy and cancerous cells were divided into concentration of 200,000 cells/mL and stored at 37 degrees Celsius. A fluorodish was plated with 25 microliters of Poly-L-Lysine (PLL) and PLL was spread over the surface. The fluorodish was placed in a sterile cell culture cabinet for five minutes to allow for maximal binding of PLL. Cells were then plated directly onto the fluorodish, and stored at 37 degrees Celsius for ten minutes to allow for maximal electrochemical adherence of cells to the surface of the fluorodish. Cells were then viewed under an optical microscope to confirm adherence. Fluorodishes were obtained from World Precision Instruments.

Atomic Force Microscopy

An MFP-3D Atomic Force Microscope (AFM) from Asylum Research was used to take measurements on Elastic Modulus and Viscoelasticity. Silicon Nitride cantilevers were placed onto the AFM head and secured with a magnetic Phillips Head Screwdriver. The AFM head was then placed over a fluorodish containing 3 ml of deionized water, and cantilevers were then visualized in both a top-view and bottom-view camera. A laser was focused onto the tip of the cantilever at a maximal x-and-y position, and the cantilevers were engaged at a -13 degree angle from the horizon. Once the cantilevers were in contact in glass, a single force measurement was taken, and the spring constant and virtual deflection line were calibrated using Asylum Research software. The deflection was set to zero. Thermal capture was utilized to account for thermal noise and intermolecular movement experienced by the cantilever due to the surroundings of the room. The fluorodish was then removed, and replaced with the fluorodish with cells to be

measured. The cantilever would then indent the surface of a cell with a specified force distance, trigger point, and downwards velocity. Once in contact, the cantilever would record a graph of cantilever deflection vs. time and force vs. time, collecting elastic data. The cantilever would then slowly retract from the cell for a specified amount of dwell time, and record viscous data. A Nikon microscope was then used to take an image from the bottom-side camera of the cell post-measurement. Measurements were repeated until ideal sample size was reached.

Analysis

Once experimentation was complete, the force vs. time curves were pulled from the Asylum Research file and were fitted with a double exponential curve fitting algorithm to find best fit of elastic modulus, and tau1 and tau2. Values that were not able to be understood by the Igor curve fitting algorithm were not recorded, and testing was repeated until sample size was reached. Images were analyzed with ImageJ to match the known diameter of the cantilever to a pixel ratio, and cell size was then recorded. Data was saved in Microsoft excel sheets, and box plots, 2D, and 3D plots were generated using Matlab.

CHAPTER 3

RESULTS

Elastic Modulus

Single force stiffness measurements were used to find the Elastic Modulus of Jurkat, HL60, K562, neutrophils, and lymphocytes. Jurkat showed an elastic modulus of 118.52 +/- 134.73. HL60 showed an elastic modulus of 372.92 +/- 328.63. K562 showed an elastic modulus of 375.54 +/- 265.88. Isolated neutrophils showed an elastic modulus of 199.49 +/- 175.49. Lymphocytes showed an elastic modulus of 601.33 +/- 386.19. These values are consistent with previous studies [2,3].

Viscoelasticity

Single force stiffness measurements with a 5 second dwell time obtained a retraction curve to measure viscous response in a quick-relaxation parameter (tau1) and a slow-response parameter (tau2). Jurkat gave a tau1 of .1676 +/- .0593 and a tau2 of 1.8847 +/- 1.1794. HL60 gave a tau1 of .2001 +/- .1761 and a tau2 of 1.993 +/- 1.5173. K562 gave a tau1 of .1725 +/- .0744 and a tau2 of 2.246 +/- 2.0209. Isolated neutrophils gave a tau1 of .1658 +/- .061 and a tau2 of 1.8756 +/- .9405. Lymphocytes gave a tau1 of .1595 +/- .0524 and a tau2 of 1.6764 +/- .5376. These values are consistent with previous studies [2,3].

Cell Diameter

Cell size was determined with relative pixel-to-unit measurement analysis for each cell. Jurkat showed a cell diameter of 11.764 +/- 1.3847. HL60 showed a cell diameter of 14.974 +/- 3.6134. K562 showed a cell diameter of 16.025 +/- 25534. Neutrophils showed a cell diameter of 13.267 +/- 2.2266. Lymphocytes showed a cell diameter of 8.4417 +/- .9736. These values are consistent with previous studies [2,3].

Differentiation Plots

An analysis of these values in Matlab yielded differentiation 2-and-3-D plots (Figures 2-8). Lymphocytes and Jurkat cells showed particular separation on the axes of cell diameter and elastic modulus, and somewhat on the tau1 axis (Figure 6). HL60, K562, and neutrophils showed particular separation on the axes of tau1 and cell diameter (Figure 4). Separation differentials for all cell types on the axes of cell diameter, elastic modulus, and tau1 can be seen in Figure 5.

CHAPTER 4

DISCUSSION

AML and CML vs. Myeloid Cells

When comparing AML leukemia with its healthy WBC counterpart, HL60 mechanical parameters were compared against healthy myeloid cells (neutrophils). Neutrophils were softer than both HL60 and K562. Neutrophils also showed smaller quick- and slow-relaxation time constants. K562 cells had significantly higher tau1 and tau2 values than both other cell types (Figure 2). Both cancerous cells were larger than the healthy myeloid cells, and K562 cells were significantly larger than both types. Neutrophils were smaller and softer, which may seem counterintuitive; this suggests that if the cytoskeletal area is equal in amount and neutrophils experience a lower cytoskeletal density, the cancer morphologies experience cytoskeletal stiffening. If this is not the case, then cytoskeletal features may be diminished altogether, or another organelle may contributing to cellular stiffness.

ALL vs. Lymphatic Cells

When comparing ALL leukemia with its healthy cell counterpart, Jurkat mechanical parameters were analyzed against lymphocytes. In contrast to the myelogenous leukemias, Jurkat was drastically softer than its healthy WBC counterpart. Jurkat and lymphocytes had virtually indistinguishable quick-response viscoelasticity time constants, however, and very similar slow-response constants. This is certainly curious; this suggests that viscosity may not be the driving mechanical property physiologically for this morphology; rather, stiffness plays a key role. As was the case in the myelogenous leukemias, the Jurkat cells were significantly smaller than their healthy lymphocytic counterparts.

Cancer Cell Types

Elastic modulus values amongst the cancer types were relatively consistent. They were much lower than lymphocytic cells, all though a hierarchy did arise; K562 were stiffest, followed by HL60, and finally the lymphocytic cancer Jurkat. The same trend was seen in cell size, following the same order with the same three cell types. Viscoelastic time constants for all three types were relatively similar. Tau2 followed the same trend as size and stiffness. The three of these in conjunction with each other may prove to lend a key role in 3-dimensional differentiation, or perhaps even 2-D. (Figure 4).

Limitations

Limitations of the study center around the subjective nature of comparative analysis. While absolute values for each type of cancer and WBC cell type are ideal, deviations in physiologic samples cause wide ranges of values for all mechanical parameters. In the cancerous morphologies, size seems to be subject to larger deviations (particularly in the K562 cell line). In healthy cells, mechanical properties seem to vary more. Cells at different phases of proliferation, differentiation, and cell cycle all may account for variances and irregularities. Because of these larger deviations, clustering analysis is more complex and subject to error. It is certainly possible on a 2-Dimensional and even 3-Dimensional axis plot to have overlap of cell types. Probability of a cell being a certain type will never reach 1.00; rather, a probability may more usefully be determined of which cell type a certain sample is not. While certain trends are evident just by visualizing the plots generated, a more sophisticated algorithm may lend in determining the exact probability that a sample of cells is indeed a given type.

CHAPTER 5

CONCLUSIONS AND APPLICATIONS

Typing Library and Microfluidics

In the future, if a library was compiled that gave each type of leukemia cell a unique mechanical profile, one could test the mechanical properties of a person's WBCs for diagnostic purposes. Similar to a CBC blood test or a peripheral blood smear, a single sample of blood could be collected and analyzed. Unlike the two former tests, if a reliable library was developed, there may not be further need for a bone marrow biopsy procedure.

Utilizing different microfluidic platforms could yield pure populations of cancer cells. Microfluidic chips used in cellular separation would be able to filter out cancer morphologies from the rest of the blood, giving clinicians a pure population of cells to run more specialized tests on for diagnostic purposes.

Cellular Staining

It is well documented that different compartments of the cell exhibit different mechanical properties [5,6]. The main organelle associated with structural rearrangement is the cytoskeleton, composed of many varying filament types. Many of these rearrangement processes cause conformational changes of the cell, and it is thought that they may play a role in the mechanical properties of cells. Numerous chemical stains exist to allow visualization of the cytoskeleton under fluorescent light. By staining the cytoskeleton and then measuring mechanical properties, one may be able to draw correlations between the physical arrangement of the structural components of the cell and cellular stiffness. Rearrangement of the cytoskeleton after perturbation may be of interest when studying viscoelastic relaxation patterns.

Chemotherapy and Drug Delivery

The addition of chemotherapy and other cancer-fighting drugs could be of interest when studying mechanical properties. Many cellular processes, including proliferation and apoptosis, can be mediated or influenced by mechanical properties, as well as cause changes in the cellular mechanics themselves. The addition of chemotherapy agents and other drugs before measurement with AFM could shed light on the mechanical properties of drug-treated leukemias.

APPENDIX A

DESCRIPTION OF DEFAULT SUBHEADING SCHEME

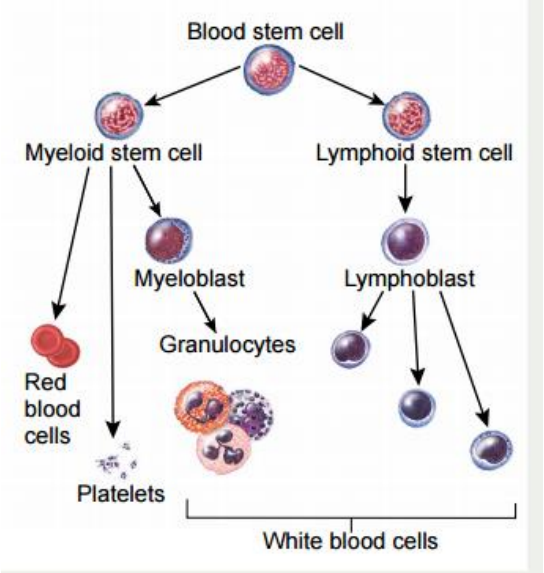

Figure 1: White Blood Cell Differentation. Taken from cancer.gov. Figure shows the progression of cells from stem cells to blasts to final cell type.

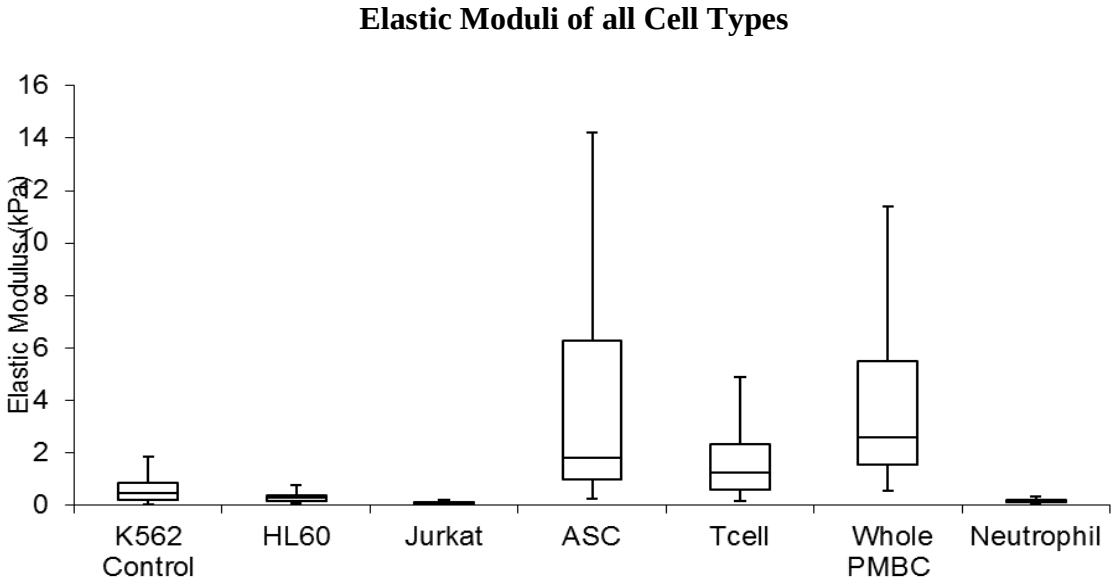

Figure 2: Elastic Moduli of all Cell Types. Box and whisker plots of each cell type is plotted in kPa.

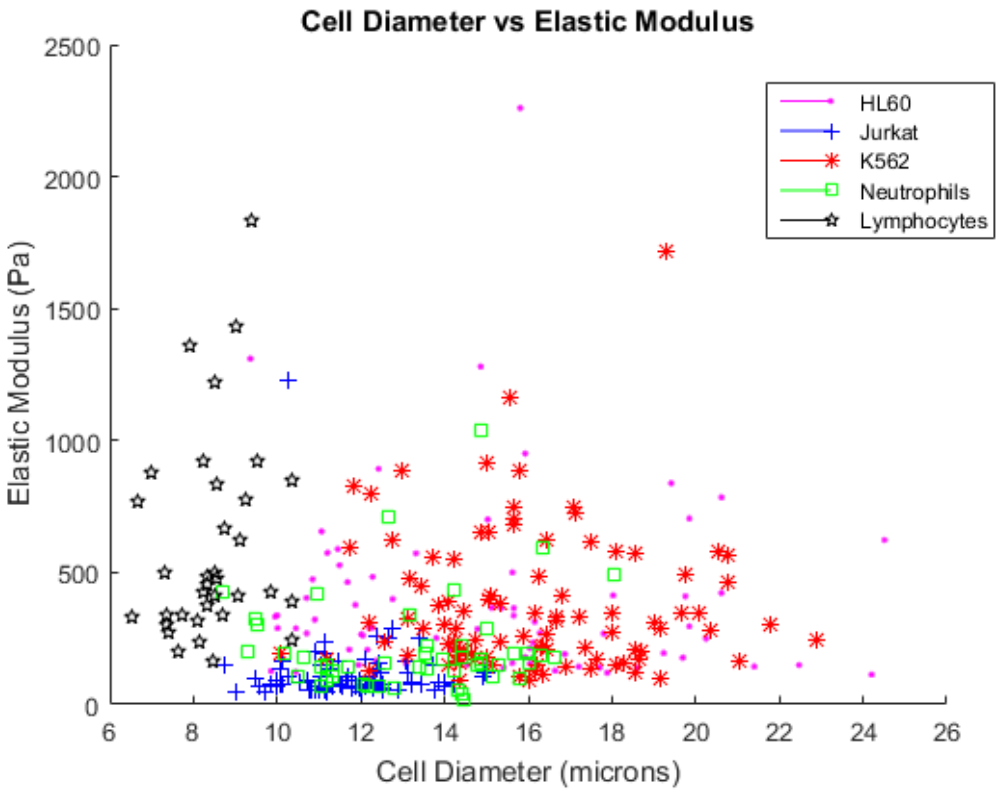

Figure 3: Elastic Modulus vs. Cell Diameter. All cell types were plotted with different markers, with elastic modulus on the y-axis and cell diameter on the x-axis.

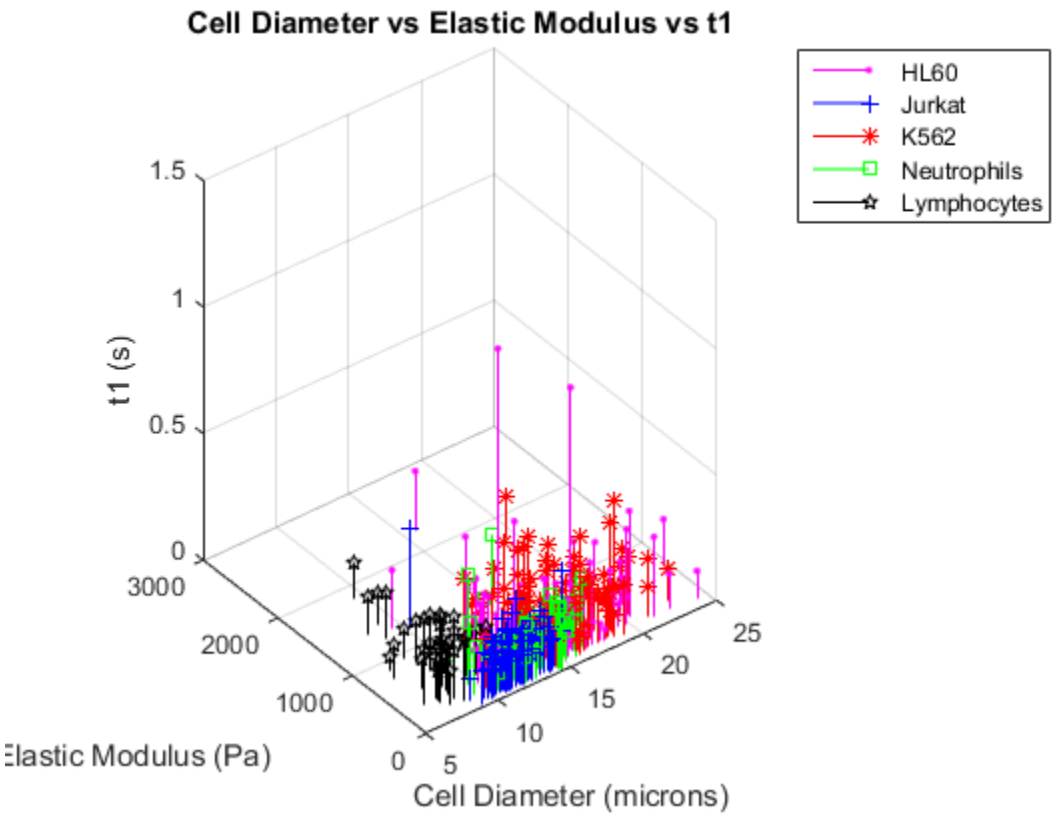

Figure 4: Cell Diameter vs. Elastic Modulus vs. tau1. All cell types were plotted with different markers, with tau1 on the z-axis, elastic modulus on the y-axis and cell diameter on the x-axis.

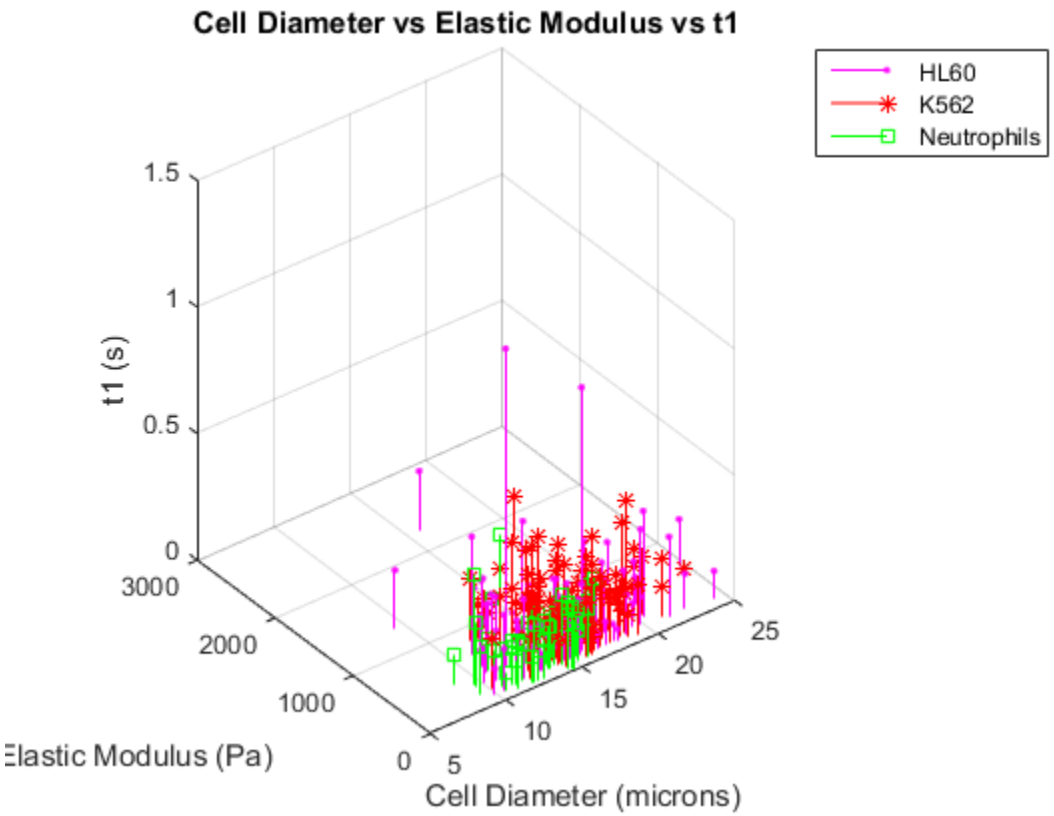

Figure 5: Cell Diameter vs. Elastic Modulus vs. tau1 for Myeloid Morphologies. Neutrophils, HL 60 and K562 cell types were plotted with different markers, with tau1 on the z-axis, elastic modulus on the y-axis and cell diameter on the x-axis.

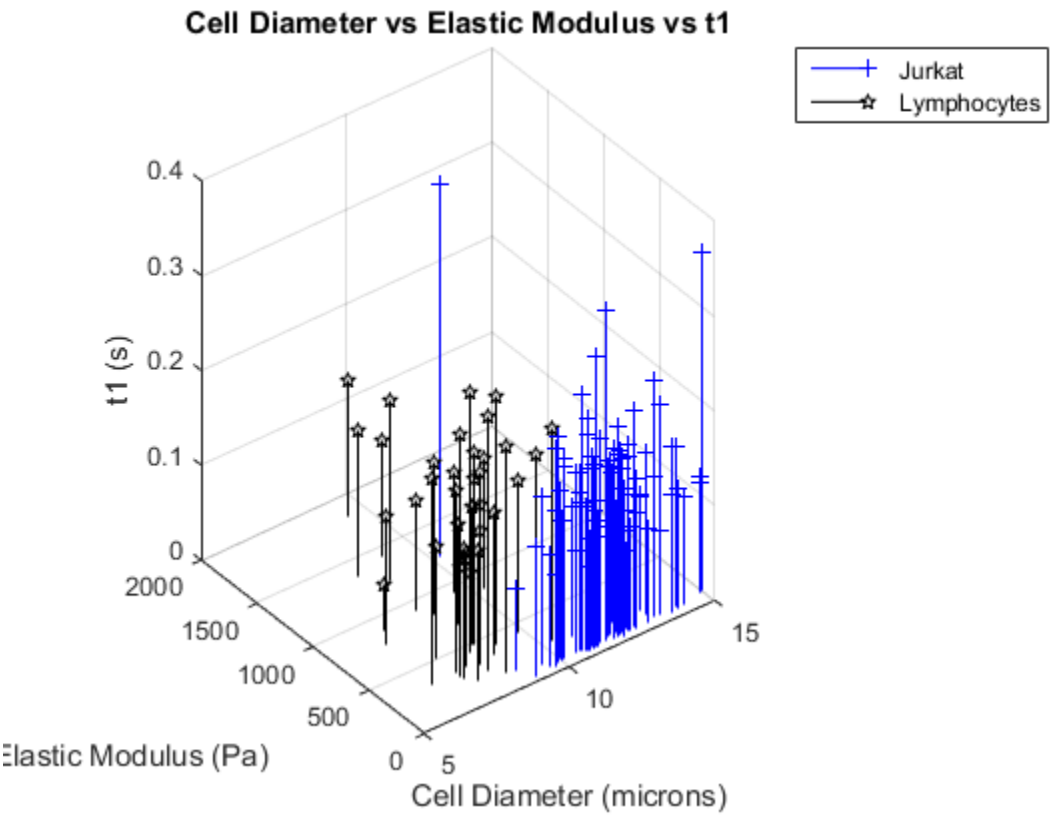

Figure 6: Cell Diamter vs. Elastic Modulus vs. tau1 for Lymphocytic Morpholoies. Lymphocytes and Jurkat cell types were plotted with different markers, with tau1 on the z-axis, elastic modulus on the y-axis and cell diameter on the x-axis.

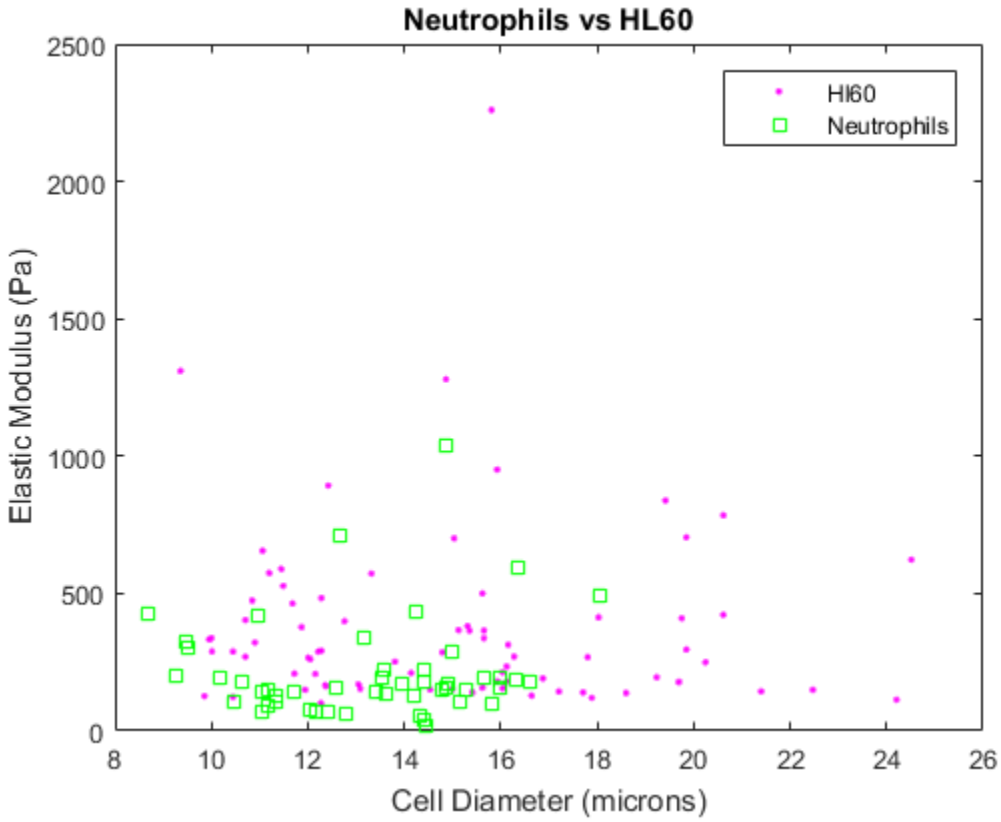

Figure 7: Elastic Modulus vs. Cell Diameter for Neutrophils and HL60 Cells. Neutrophils and HL60 cell types were plotted with different markers, with elastic modulus on the y-axis and cell diameter on the x-axis.

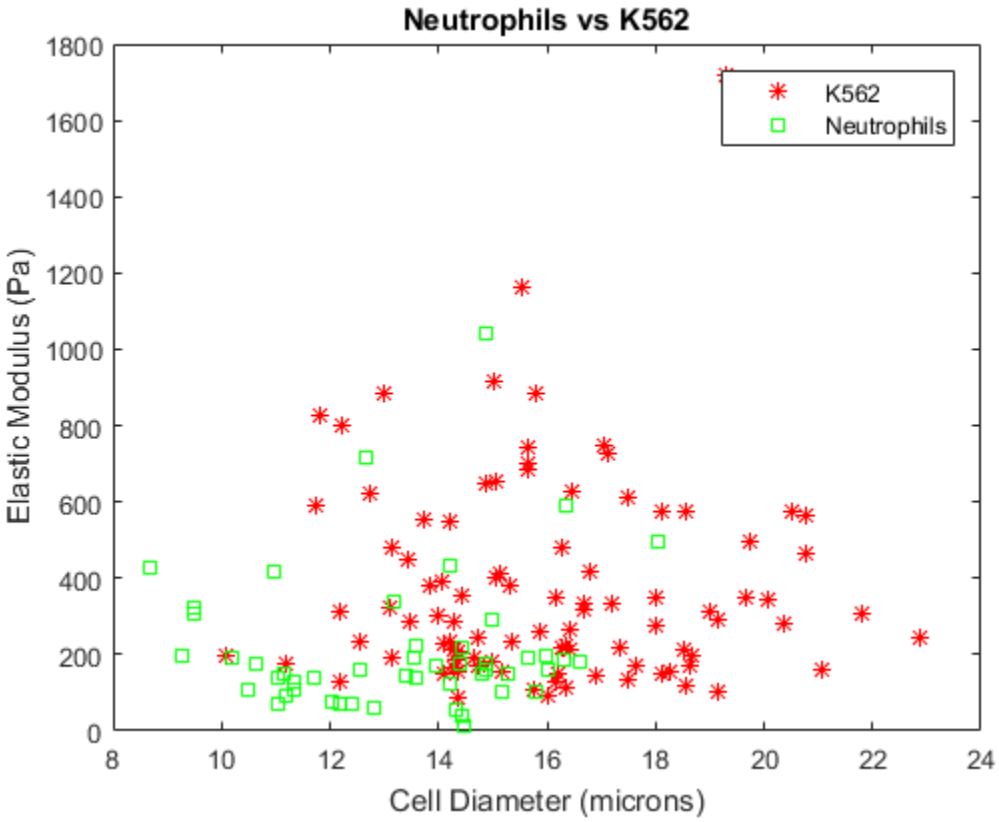

Figure 8: Elastic Modulus vs. Cell Diameter for Neutrophils and K562 Cells. Neutrophils and K562 cell types were plotted with different markers, with elastic modulus on the y-axis and cell diameter on the x-axis.

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